METHODOLOGICAL ASPECTS AND APPLICATIONS OF FLUORESCENT *IN SITU* HYBRIDIZATION TO IDENTIFY BACTERIA FROM THE GASTROINTESTINAL TRACT OF TURKEYS

Agnieszka Skowrońska[1], Dariusz Mikulski[2], Marzena Mikulska[2], Piotr Zapotoczny[3], Izabela Zmysłowska[1], Piotr M. Szczypiński[4]
[1]*Department of Environmental Microbiology, Faculty of Environmental Protection and Fisheries, Warmia and Mazury University, Prawocheńskiego 1, 10-718 Olsztyn, Poland*
[2]*Department of Poultry Science, Faculty of Animal Bioengineering, Warmia and Mazury University, Oczapowskiego 5, 10-719 Olsztyn, Poland*
[3]*Department of Agricultural Process Engineering, Faculty of Technical Sciences, Warmia and Mazury University, Heweliusza 14, 10-718 Olsztyn, Poland*
[4]*Institute of Electronics, Technical University, Wólczańska 211/215, 90-924 Łódź, Poland*

Summary. The microflora of avian gastrointestinal tract has received increased attention as the focus of efforts to improve animal nutrition and to minimize food-borne illness in humans. This microflora is subjected to variations according to diet, environment and treatments. Conventional methods for determining the flora composition rely on the cultivation of bacteria on anaerobic selective media. This approach is time-consuming because many bacteria are difficult to culture or are uncultivable. Molecular tools introduced in microbial ecology have made it possible to study the composition of intestinal flora in a culture-independent way. Our paper describes a methodological aspects and application of FISH method with rRNA-targeted fluorescent probes to identify bacteria from the gastrointestinal tract of turkey. This technique poses advantages compared with traditional culture-based methods as it does not require cultivation of the target organisms. FISH enables to address simultaneously various ecological issues: 1. to by-pass cultivation problems, 2. to identify and to accurately enumerate prokaryote sub-populations in natural systems by using sets of probes 3. to analyze their spatial distributions. Although FISH has been used widely in bacterial ecology, the method requires some adaptation to the type of samples analyzed. Applying the optimized FISH method for the analysis of turkey caecum, we were able to identify *Eubacterium rectale*, the genera *Enterobacteriaceae*, *Bacteroides, Bifidobacterium*, and *Lactobacillus-Enterococcus* groups.

Keywords: bacteria, oligonucleotide probes, 16S rRNA, FISH, caecum, turkey.

FLUORESCENCINĖS HIBRIDIZACIJOS *IN SITU* (FISH) METODOLOGINIAI ASPEKTAI IR PRITAIKYMAS KALAKUTŲ VIRŠKINAMOJO TRAKTO BAKTERIJOMS IDENTIFIKUOTI

Agnieszka Skowrońska[1], Dariusz Mikulski[2], Marzena Mikulska[2], Piotr Zapotoczny[3], Izabela Zmysłowska[1], Piotr M. Szczypiński[4]
[1]*Aplinkos mikrobiologijos katedra, Aplinkosaugos ir žuvininkystės fakultetas, Varmijos-Mozūrijos universitetas, Pravochenskiego 1, 10-718 Olštinas, Lenkija*
[2]*Paukštininkystės katedra, Gyvūnų bioinžinerijos fakultetas, Varmijos-Mozūrijos universitetas, Oczapovskiego 5, 10-719 Olštinas, Lenkija*
[3]*Agrotechnikos katedra, Technikos mokslų fakultetas, Varmijos-Mozūrijos universitetas, Heveliusza 14, 10-718 Olštinas, Lenkija*
[4]*Elektronikos institutas, Technikos universitetas, Wolczanska 211/215, 90-924 Lodzė, Lenkija*

Santrauka. Pastebėta padidėjęs domėjimasis paukščių virškinamojo trakto mikroflora – norima pagerinti gyvūnų mitybą ir sumažinti maisto produktų sukeltų žmogaus ligų riziką. Mikroflora gali kisti priklausomai nuo lesalo, aplinkos ir taikomų procedūrų. Įprastiniai floros sudėties nustatymo metodai pagrįsti bakterijų auginimu atrankinėse anaerobinėse terpėse. Jie reikalauja daug laiko, nes daugumą bakterijų kultūrų sunku auginti arba jų pasėliai visai nesidaugina. Molekulinės tyrimo priemonės, įdiegtos mikroorganizmų ekologijoje, leidžia tyrinėti žarnyno floros sudėtį be bakterijų pasėlių. Straipsnyje aprašomi FISH metodo su fluorescenciniais žymekliais, orientuotais į rRNA, metodologiniai aspektai ir pritaikymas identifikuoti kalakutų skrandžio bei žarnyno trakto bakterijas. Šis būdas turi privalumų palyginti su įprastais bakterijų pasėliu pagrįstais metodais, nes tiriamų organizmų nereikia dauginti. FISH leidžia vienu metu spręsti skirtingus ekologinius klausimus: 1. apeiti bakterijų dauginimo problemas; 2. panaudojant žymeklių rinkinius identifikuoti ir tiksliai apskaičiuoti prokariotinių ląstelių pogrupius natūraliose sistemose; 3. išanalizuoti jų erdvinį pasiskirstymą.

Nors FISH metodas plačiai taikomas bakterinėje ekologijoje, jį reikia pritaikyti konkretiems analizuojamiems mėginiams. Pritaikę FISH metodą kalakutų aklosios žarnos analizei, mes galėjome identifikuoti *Eubacterium rectale*, *Enterobacteriaceae*, *Bacteroides, Bifidobacterium* gentis ir *Lactobacillus-Enterococcus* grupes.

Raktažodžiai: bakterijos, oligonukleotidų žymekliai, 16S rRNR, FISH, akloji žarna, kalakutai.

Introduction. In modern poultry production, breeding measures which guarantee adequate development and functioning of the gastrointestinal tract are required to keep the birds in good health. Nutrition is the main factor which determines the host's resistance to bacteria colonizing the alimentary tract. Adequate and fully active gastrointestinal microflora stimulates the immune system, protects the host from intestinal infections and improves the assimilability and utilization of feed nutrients (Mead, 1997; Vispo, Karasov, 1997; van der Wielen et al., 2002). The importance of microflora of the avian gastrointestinal tract has been recognized in recent research studies to determine the effectiveness of various biostimulators (including oligosaccharides, ß-glucan, priobiotic bacteria and essential oils) with the aim of stimulating the activity of beneficial bacteria and controlling the proliferation of pathogenic bacteria (Apajalahti et al., 2001, 2004; Mitsch et al., 2004; Immerseel et al., 2005; Mikulski et al., 2008).

The gastrointestinal tract of animals is colonized by a highly complex and diversified microbial community comprising several hundred types of bacteria, mostly obligate anaerobes of the domain *Bacteria*. Dietary nutrients provide gastrointestinal bacteria with the energy required for growth and proliferation. Their nutritional requirements vary, which is why the chemical composition and components of gastric contents largely determine the spatial distribution of different bacteria in the alimentary tract. The process of isolation and biochemical differentiation of bacteria is difficult to perform in an anaerobic environment for technical reasons. Despite numerous research efforts, adequate conditions supporting *in vitro* growth have not yet been developed for most bacteria (Amann et al., 1995). In the natural environment of the gastrointestinal tract, bacterial communities are highly diversified populations of interdependent bacterial strains. Conventional medical and veterinary diagnostic methods based on an analysis of phenotypic characters (morphological, biochemical, serological) are insufficient for effective identification of bacteria. The above is largely due to the phenotypic diversity of bacteria. The process of culturing a sufficient bacterial population for this type of analyses is also time-consuming. The main limitation of growing methods is posed by the fact that only 10 to 60% of bacteria isolated from the gastrointestinal tract of broiler chickens are capable of *in vitro* growth (Barnes et al., 1972, 1979; Salanitro et al., 1974). On the other hand, microbial populations which are successfully cultured in a laboratory do not always behave in the same way as in their natural environment where the availability of nutrients is limited and mutual metabolic interactions are observed between bacteria.

Molecular biology methods (Table 1) relying on genetic material analyses were introduced to conventional microbiological practice in the late 1980s. The PCR method, PCR-rRNA cloning and sequencing generated information on the structure of microbial communities from various environments. Today, universal and specific primers enable rRNA gene amplification from potentially every bacterial sample, omitting the culture stage. The amplified region can be then sequenced and compared with the sequences deposited in the GenBank (http://ncbi.mnl.nih.gov/blast/cgi). The sequencing strategy has been universally applied to compare rRNA gene sequences from various microbial communities based on variable regions, and to identify the resulting affinity. The rRNA sequences complementary to 16S rRNA and 23 rRNA genes facilitated the design of probes for identifying bacteria from both the isolated microbe as well as those known solely by the sequence. The number of deposited bacterial sequences continues to increase, and it supports the subsequent design of specific probes.

Table 1. **Comparison of currently applied methods for analyses of microbial communities**

Method	Usefulness of method	Limitations
Growing microbial cultures	possibility of isolation, "ideal"	- non-representative; - time-consuming and laborious analyses
16S rDNA sequencing	phylogenetic identification	- incomplete lysis of selected microbes in a given population; - not all rRNA genes are equally amplified; - time-consuming interpretation of sequencing results
DGGE/TGGE/TTGE	- *monitoring sudden changes in microbial populations;* - fast comparative analyses	inhibitors in PCR sample; semiquantitative method; - identification requires cloning analyses and access to a clone library
FISH	- count of microorganisms of a given type in a sample; - quantitative method; - automated image analysis; - physical association of various microorganisms	- incomplete permeabilization of selected bacteria; - environment-specific probe; - information on various microbial sequences is required

Fluorescent in situ hybridization (FISH) is one of the most advanced molecular biology methods which are popularly applied in both environmental and medical microbiology (Amann et al., 1995; Moter, Gobel, 2000). The FISH method is used to detect target sites in the RNA sequence of various microbes (including both cultured

microorganisms and those known solely by the sequence) with the use of fluorescent labeled probes. Based on the sequence of the 16S rRNA gene, molecular probes are developed in the form of DNA fragments (rDNA probes) which are capable of hybridizing specifically with rRNA and rRNA genes of the given bacterial type, genus species or strain. Probes may also consist of short gene fragments encoding the synthesis of toxins and hemolysins which determine the pathogenicity and antibiotic resistance of an organism (Zakrzewska-Czerwińska, 2002). The FISH method also indicates whether the sequences obtained by PCR/sequencing are represented in the investigated environment. As an additional advantage, the method enables precise enumeration of microbial populations and their physical association in diverse communities. FISH does not require DNA isolation from the studied microorganism because cells are immobilized in a solid medium. Fast-growing cells contain a sufficient number of ribosomes (10^3-10^5), producing a strong signal when a labeled probe binds to the rRNA of a single cell (Figure 1).

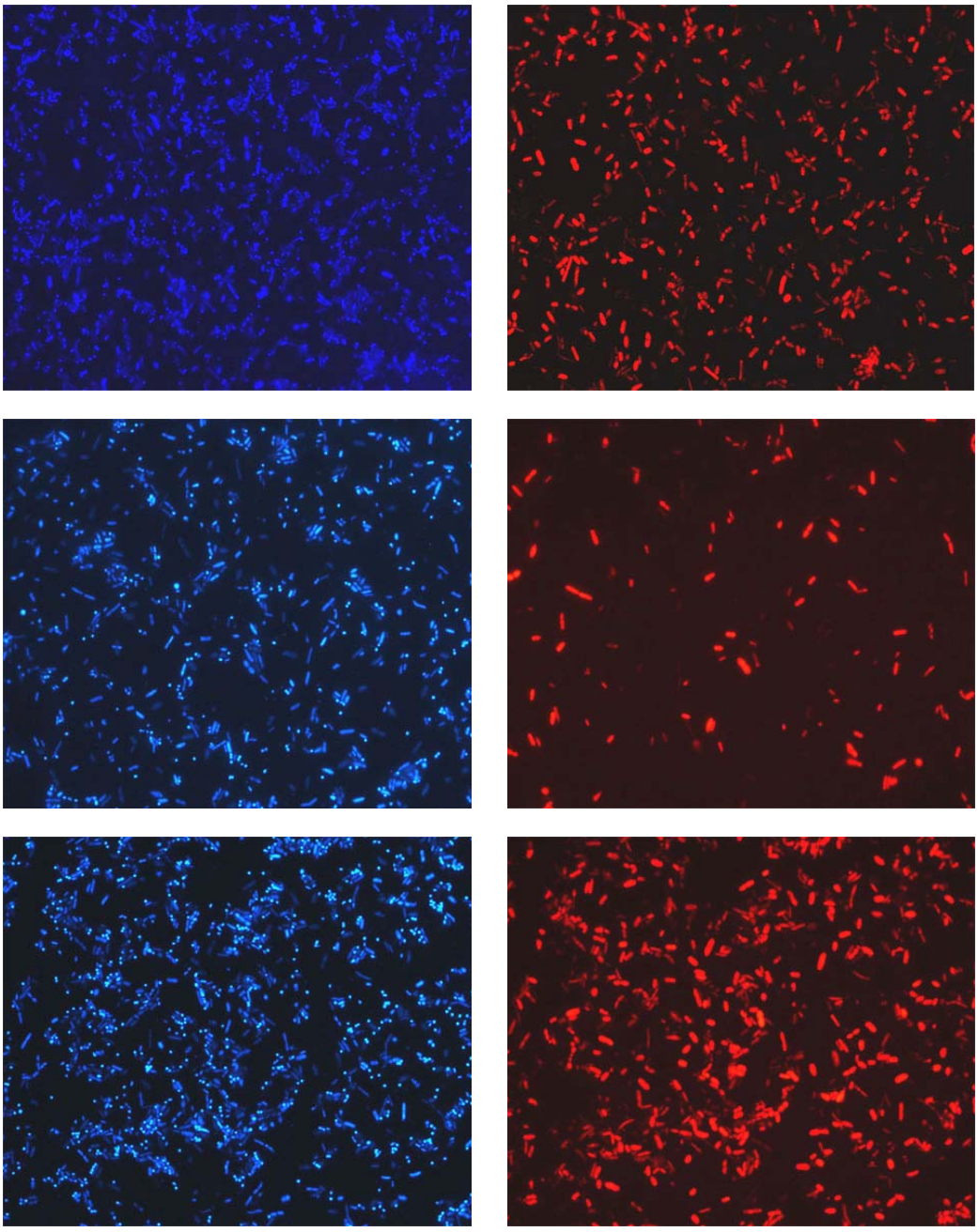

Figure 1. **Examples of hybridization images:** A – Eub338 probe identifying all bacteria of the domain *Bacteria*, B – Erec482 probe identifying *Clostridium coccoides* and *Eubacterium rectale*, C – Lab158 probe identifying *Lacobacillus-Enterococcus*. Left side – DAPI image – blue-stained bacteria. Right side – post-hybridization image – red-stained bacteria

The aim of our study was to apply the FISH method. Our paper describes a methodological aspects and application of FISH method with rRNA-targeted fluorescent probes to identify bacteria from the gastrointestinal tract of turkey.

Material and methods. In own study (manuscript under preparation) of the cecal digesta of 8-week-old turkeys, the authors applied the membrane filter method to determine the population size of selected bacterial groups: *Bacteroides, Clostridium coccoides-Eubacterium rectale, Enterobacteriaceae, Lactobacillus-Enterococcus* and *Bifidobacterium.*

Samples of cecal digesta (approximately 1.5 g) were diluted with sterile physiological saline and placed in a stomacher (Stomacher BagMixer 400 Interscience). The supernatant was collected after stomaching and fixed with paraformaldehyde for 12h at 4ºC. The fixed bacterial suspension was centrifuged (12 000 rpm for 10 minutes), bacteria were deposited on white 0.2 µm polycarbonate filters (Millipore) by filtration with the use of sterile filtration devices (Nalgene). Filters were then stained with DAPI dye (Porter, Feig, 1980). A thick bacterial suspension with local biofilm conglomerates was observed under a fluorescent microscope. Numerous bacterial colonies prevented an effective microbial count. The suspension was further diluted (1:150) to improve microbial count effectiveness, and an adequate quantity of the bacterial suspension was deposited on white polycarbonate filters. 8.0 µm membrane filters (Sartorius) were additionally used during filtration to uniformly distribute bacteria on the filter. Filters were rinsed with sterile distilled water and excess water was removed by gentle filtration. Filters were thoroughly dried and stored in Petri dishes at -20ºC for further analyses.

The hybridization procedure was conduced in accordance with the method described by Fuchs et al. (2007) and Langendijk et al. (1995). Filters were cut into sections and the buffer was prepared for hybridization (900mM NaCl, 20mM Tris/HCl [pH 7.4], 0.01%SDS, formamide, deionized water). Probes were labeled with Cy3 cyanine dye at the 5' terminal end (Thermo ElectronGmbH, Germany). The hybridization mix comprising the hybridization buffer and a probe (50 ng μl^{-1}) was stored on ice. Filters were placed on slides and the buffer with the probe were applied. Filters were placed horizontally in Falcon test tubes and the remaining hybridization buffer was added. Hybridization was carried out in a darkened incubator. As suggested by reference data, formamide concentration, hybridization temperature and incubation time were modified subject to the type of the applied probe. To identify Gram(+) bacteria, lysozyme diluted in the TE-HIS buffer (100mM Tris-HCL [pH 8.0], 50mM EDTA) was applied and was incubated for 10 minutes at room temperature. Rinse buffer (900mM NaCl, 20mM Tris/HCl, 50mM EDTA [pH 8.0], 0.01%SDS, deionized water) was prepared after hybridization and filters were rinsed for 15 - 20 minutes in a water bath. Similarly to hybridization, the temperature of the rinse buffer and rinsing time were modified subject to the type of probe. Then the filters were rinsed with sterile deionized water (80% ethanol), and dried thoroughly. Dried filters were stained with DAPI dye according to the method proposed by Porter and Feig (1980), they were moisturized with a combination of Citifluor-Vectashield oils and glued to slides. Stained bacterial cells were viewed under a fluorescent microscope (Olympus BX 51) equipped with a set of filters for DAPI (Ex 330 – 380 nm, DM – 400 nm, BA – 420 nm) and CY3 (Ex 450 – 490 nm, DM – 505 nm, BA – 520 nm). 20 photographs of each filter were taken from random fields for both DAPI and hybridized bacteria (Figure 1). The authors relied on the modified version of the MaZda 4.5 application (Institute of Electronics in Łódź) to count bacteria in the DAPI image, which showed all bacteria stained light blue, and in the post-hybridization image, where the identified bacteria were stained red (Fig. 1).

Discussion. In 1988, Stahl et al. (1988) were the first to demonstrate that 16S rRNA-targeted probes can be applied in the bovine gastrointestinal tract without resorting to culture methods. They used specific oligonucleotide probes identifying the strains *Fibrobacter succinogenes* and *Lachnospira multiparus* in the alimentary tract of cattle.

As regards bacteria identified in the gastric contents of birds, a typical protocol has to be changed and optimized. Sampled digesta should be thoroughly homogenized or stomached. This is very important because only a small quantity of gastric contents is to be analyzed at subsequent stages, which is why the collected sample has to be representative. Undigested residues and other particles are removed by short centrifugation (<300 g). A similar procedure is adopted regardless of the technical hybridization variant, and it comprises the following stages: (a) initial preparation of samples, including stomaching, homogenization, centrifuging and fixing (Manz et al., 1995; Langendijk et al., 1995; Barc et al., 2003), (b) for the identification of Gram(+) bacteria – the use of enzymes (e.g. lysozyme, achromopeptidase, lipase) for cell wall digestion, (c) hybridization with a selected probe, (d) washing to remove the unbound probe, (e) viewing the identified bacteria under a fluorescent or confocal microscope or by flow cytometry, and documenting the results.

Published data indicate that most research studies rely on the FISH method combined with fluorescence microscopy (Langendijk et al., 1995; Zoetendal et al., 2002). When combined with flow cytometry (Wallner et al., 1993; Barc et al., 2003), those methods can be deployed for multiparametric analyses and relatively rapid and sensitive quantitative analyses of bacterial populations, including those with a percentage share of less than 1%.

The correct fixing of biological material is one of the prerequisites for effective hybridization *in situ*. Prior to hybridization, bacteria should be fixed to account for the permeabilization stage, i.e. a process which facilitates probe insertion into a cell. It preserves the integrity and morphological structure of cells and protects RNA from degradation caused by the presence of endogenous RNases. Precipitation fixatives (ethanol, methanol) or cross-linking fixatives (formalin, paraformaldehyde) may be applied. Fixing conditions may differ subject to the

type of sample, the probe and the planned method of visualizing the results. Effective fixing has a decisive impact on the visualization of bacteria and is difficult to optimize. Most authors believe 3-4% formaldehyde or paraformaldehyde is the optimal fixative because it facilitates the identification of Gram(-) bacteria. Gram(+) bacteria may be identified with the use of 50% ethanol or ethanol/formalin (9:1).

An optimal hybridization environment (temperature, salt [monovalent cation] concentration in the hybridization mix, incubation time and formamide concentration) should be created to maximize reaction specificity. In most cases, formamide concentrations higher than 50% are not applied because reaction rate decreases as formamide concentration increases. Hybridization takes place in a darkened incubator at a temperature of 37ºC to 50ºC. Most 16S rRNA-targeted probes applied in FISH are oligonucleotide probes containing from 15 to 25 nucleotides (Table 2). The time required for the formation of complementary hydrogen bonds between the probe and the studied rRNA fragment ranges from 30 minutes to several hours. Reaction sensitivity is also determined by the type of fluorescent tracer which is covalently bonded to the probe at the 5' terminal end. The most popularly applied probe labels are fluorescein, tetramethylrhodamine, Texas Red and Cyanine-3. Their emission spectra are a measure of signal intensity emitted by every hybridized cell. Proper hybridization may be conducted with the use of membrane filters (Pernthaler et al., 2002) and gel-coated slides (Manz et al., 1992; Fuchs et al., 2007). Both methods may be successfully applied to identify gastrointestinal bacteria.

Table 2. **Oligonucleotide probes used for the identification of bacteria isolated from the gastrointestinal tract**

Probe	Probe specificity	Probe sequence (5' - 3')	References
Eub338	*Bacteria* without Planctomycetales	GCTGCCTCCCGTAGGAGT	Amann et al., 1990
Non388	Nonsense probe for identification	ACTCCTACGGGAGGCAGC	Wallner et al., 1993
Bif164	*Bifidobacterium*	CATCCGGCATTACCACCC	Langendijk et al., 1995
Enter1433	*Enterobacteriaceae*	GTTTTGCAACCCACT	Sghir et al., 2000
Bac1080	*Bacteroides*	GCACTTAAGCCGACACCT	Dore et al., 1998
Erec482	*C.coccoides-E. rectale*	GCTTCTTAGTCAGGTACCG	Franks et al., 1998
Lab158	*Lactobacillus-Enterococcus*	GGTATTAGCA(C/T)CTGTTTCCA	Harmsen et al., 1999
Chis150	*Clostridiaceae*	TTATGCGGTATTAATCTYCCTTT	Franks et al., 1998
Eco1531	*Enterobacteriaceae* spp	CACCGTAGTGCCTCGTCATCA	Polusen et al., 1993
Unk730	Unidentified intestinal bacteria, DF1	TAGAGCCCAGTAAACCGC	Huber et al., 2004

Computer-aided image analysis is becoming an increasingly popular tool in medical microbiology. A fluorescence microscope should be equipped with efficient analyzing software, sensitive CCD cameras and at least three sets of fluorescence filters for observation at the most popular light, excitation and emission wavelengths (blue/green – fluorescein, UV/blue – DAPI, green/red – rhodamine, cyanine). Quantitative and qualitative parameters of the investigated object can be identified in the image with the use of computer software. The image produced by the registering device is not always legible enough to identify objects with the use of simple image processing functions. As regards analyses of bacteria from turkey cecal contents, a vast number of objects which are displayed in photographs after hybridization and DAPI staining formed conglomerates that were difficult to count. A single photograph often contained around 1500 – 2000 bacteria (DAPI image), some of which came into contact, overlapped or were in the process of division. Several lightness maxima were observed within the contour of a single bacterium which significantly obstructed the process of computing the binarization threshold and counting. Despite the availability of filters and transformation options, standard software does not always guarantee the achievement of the set objective. If this is the case, dedicated software should be developed. The authors relied on the modified version of the MaZda 4.5 application (Institute of Electronics in Łódź) to count bacteria. The application enables the user to change the following settings – "threshold change step", "merge object step", "background intensity", "smooth object contour", "object depth" to obtain the correct segmentation threshold. The following operations support image analysis: binarization, erosion, dilation, morphological properties, median filters and histogram normalization. The program features additional options such as multiple image processing (faster count) and monitoring the correctness of the analysis by overlapping the input file with the source file.

Conclusion. To conclude, the FISH method supported direct identification, visualization and location of individual bacteria in the caecum of turkeys. It is a highly sensitive analytical technique for identifying very demanding bacteria which are difficult to culture in a laboratory. The FISH method also enables to track changes in microbial structures caused by various factors.

Acknowledgments. This study was supported by the Polish Ministry of Science and Higer Education (year 2008-2011), as a scientific project (grant number N N311 098334).

References

1. Amann R., Ludwig W., Schleifer K-H. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 1995. Vol. 59. P. 143–169.

2. Amann R.I, Binder B.J., Olson R.J., Chischolm S.W., Devereux R., Stahl D.A. Combination of 16S

rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. 1990. Vol. 56. P. 1919–1925.

3. Apajalahti J., Kettunen A., Graham H. Characteristics of the gastrointestinal microbial communities, with special reference to the chicken. World`s Poult. Sci. J. 2004. Vol. 60. P. 2223–2232.

4. Apajalahti J.H., Kettunen A., Bedford M.R., Holben W.E. Percent of G+C profiling accurately reveals diet-related differences in the gastrointestinal microbial community of broiler chickens. Appl. Environ. Microbiol. 2001. Vol. 67. P. 5656–5676.

5. Barc M.C., Bourlioux F., Rigottier-Gois L., Charrin-Sarnel C., Janoir C., Boureau H., Dore J., Collignon A. Effect of amoxicillin-clavulanic acid on human fecal flora in a gnotobiotic mouse model assessed with fluorescence hybridization using group-specific 16S rRNA probes in combination with flow cytometry. Apply. Environ. Microbiol. 2004. Vol. 48: 1365–1368.

6. Barnes E.M., Mead G.C., Barnum D.A., Harry E.G. The intestinal flora of the chicken in the period 2-6 weeks of age, with particular reference to anaerobic bacteria. Br. Poult. Sci. 1972. Vol. 13. P. 311–326.

7. Barnes E.M. The intestinal microflora of poultry and game birds during life and storage. J. Appl. Bacteriol. 1979. Vol. 46. P. 407–419.

8. Dore J., Sghir A., Hannequart-Gramet G., Corthier G., Pochart P. Design and cultivation of 16S rRNA targeted oligonucleotide probe specific detection and quantification of human fecal Bacteroides populations. Syst. Appl. Microbiol. 1998. Vol. 21. P. 65–71.

9. Franks A.H., Harmsen H.J.M., Raangs G.C., Jansen G.J., Schut F., Welling G.W. Variations of bacterial populations in human feces measured by fluorescent *in situ* hybridization with group specific 16S rRNA-targeted oligonucleotide probes. Appl. Environ. Microbiol. 1998. Vol. 64. P. 3336–3345.

10. Fuchs B. M., Pernthaler J., Amann R. Single cell identification by fluorescence *in situ* hybridization. Methods for General Molecular Microbiology Ed. 3. 2007.

11. Harmsen H.J.M., Elfferich P., Schut F., Welling G.W. A 16S rRNA targeted probe for the detection of lactobacilli and enterococci in fecal sample by fluorescent in situ hybridization. Microb. Ecol. Health. Dis. 1999. Vol. 11, 3–12.

12. Huber I., Spanggard B., Appel K.F., Rossen L., Nielsen T., Gram L. Phylogenetic analysis and in situ identification of the intestinal microbial community of rainbow trout (Oncorhynchus mykiss, walbaum). J. Appl. Microbiol. 2004. Vol. 96. P. 117–132.

13. Immerseel F.Van, Boyen F., Gantois I., Timbermont L., Bohez L., Pasmans F., Haesebrouck F., Ducatelle R. Supplementation of coated butyric acid in the feed reduces colonization and shedding of Salmonella in poultry. Poultry Sci. 2005. Vol. 84. P. 1851–1856.

14. Langendijk P.S., Schut F., Jansen G.J., Raangs G.C., Kamphuis G.R., Wilkinson M.H.F., Welling G.W. Quantitative Fluorescence in situ hybridization of Bifidobacterium spp. With genus–specific 16S rRNA-targeted probes and its application in fecal samples. Appl. Environ. Microbiol. 1995. Vol. 61. P. 3069–3075.

15. Manz W., Amann R., Ludwig W., Wagner M., Schleifer K.H. Phylogenetic oligonucleotide probes fort the major subclasses of Proteobacteria: Problems and solutions. Syst. Appl. Microbiol. 1992. Vol. 15. P. 593–600.

16. Mead G.C. Bacteria in the gastrointestinal tract of birds. Pages 216-240 in Gastrointestinal Microbiology. 2. Gastrointestinal Microbes and Host Interactions. R. J. Mackie, B. A. White, and R. E. Isaacson, ed. Chapman and Hall, New York, 1997.

17. Mikulski D., Zduńczyk Z., Jankowski J., Juśkiewicz J. Effects of organic acids or natural plant extracts added to diets for turkeys on growth performance, gastrointestinal tract metabolism and carcass characteristics. J. Anim. Feed Sci. 2008. Vol. 17. P. 233–246.

18. Mitsch P., Zitterl-Eglseer K., Kohler B., Gabler C., Losa R., Zimpernik I. The effect of two different blends of essential oil components on the proliferation of Clostridium perfringens in the intestines of broiler chickens. Poultry Sci. 2004. Vol. 83. P. 669–675.

19. Moter A., Gobel U.B. Fluorescence in situ hybridization (FISH) for direct visualization of microorganisms. J. Microbiol. Methods 2000. Vol. 41. P. 85–112.

20. Pernthaler J., Glöckner F.O., Schönhuber W., Amann R. Fluorescent in situ hybridization (FISH) with oligonucleotide probes. Methods in Microbiology 2002. Vol. 30. P. 207–224.

21. Porter K.G., Yvette S.F. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 1980. Vol. 25. P. 943–948.

22. Polusen L.K., Ballard G., Stahl D. Use of rRNA fluorescence in situ hybridization for measuring the activity of single cells in young established biofilms. Appl. Environ. Microbiol. 1993. Vol. 59. P. 1354–1360.

23. Salanitro I.G., Fairchilds J.P., Zgornicki Y.D. Isolation, culture characteristics and identification of anaerobic bacteria from the chicken caecum. Appl. Microbiol. 1974. Vol. 27. P. 678–687.

24. Sghir A., Gramet G., Suau A., Rochet V., Pochart P., Dore J. Quantification of bacterial groups within human fecal flora by oligonucleotide probe hybridiza-

tion. Appl. Environ. Microbiol. 2000. Vol. 66. P. 2263–2266.

25. Stahl D.A. Phylogenetically based study of microbial ecosystem perturbation, in Biotechnology for crop protection, Hedin, PA., Menn JJ., and Hollingsworth RM., Eds. American Chemical Society Symposium Series 379, 1988.

26. Van der Wielen P.W., Keuzenkamp D.A., Lipman L.J., van Knapen F., Biesterveld S. Spatial and temporal variation of the intestinal bacterial community in commercially raised broiler chickens during growth. Microb. Ecol. 2002. Vol. 44. P. 286–293.

27. Veldhuizen E.J.A., Tjeerdsma van Bokhoven J.M.L., Zweijtzer C., Burt S.A., Haagsman H.P. Structural requirements for the antimicrobial activity of carvacrol J. Agric. Food. Chem. 2006. Vol. 54. P. 1874–1879.

28. Vispo C., Karasov W.H. The interaction of avian gut microbes and their host: An elusive symbiosis. Pages 116–155 in Gastroinestinal Microbiology. 1. Gastrointestinal Ecosystem and Fermentations. R. J. Mackie, and B. A. White, ed. Chapman and Hall, New York, 1997.

29. Wallner G., Amann R., Beisker W. Optimizing fluorescent in situ hybridization with rRNA-targeted oligonucleotide probes for flow cytometric identification of microorganisms. Cytometry 1993. Vol. 14. P. 136–143.

30. Yamamoto T., Morotomi M., Tanaka R. Species-specific oligonucleotide probes for five Bifidobacterium species detected in human intestinal microflora. Appl. Environ. Microbiol. 1992. Vol. 58. P. 4076–4083.

31. Zakrzewska-Czerwińska J. Sondy molekularne w diagnostyce mikrobiologicznej. Biotechnologia 2002. Vol. 4. P. 79–89.

32. Zoetendal E.G., Ben-Amor K., Harmsen H.J.M., Schut F., Akkermans A.D.L., Willem M. de Vos. Quantification of uncultured Ruminococcus obeum-like bacteria in human fecal samples by fluorescent in situ hybridization and flow cytometry using 16S rRNA-targeted probes. Appl. Environ. Microbiol. 2002. Vol. 68. P. 4225–4232.

Received 28 October 2008
Accepted 7 September 2009